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Confirmational Identification of *Escherichia coli*, a Comparison of Genotypic and Phenotypic Assays for Glutamate Decarboxylase and β-D-Glucuronidase

A. E. MCDANIELS,[1]* E. W. RICE,[2] A. L. REYES,[3] C. H. JOHNSON,[2] R. A. HAUGLAND,[1] AND G. N. STELMA, JR.[1]

National Exposure Research Laboratory,[1] National Risk Management Research Laboratory,[2] and Senior Environmental Program,[3] U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

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Genotypic and phenotypic assays for glutamate decarboxylase (GAD) and β-D-glucuronidase (GUD) were compared for their abilities to detect various strains of *Escherichia coli* and to discriminate among other bacterial species. Test strains included nonpathogenic *E. coli*, three major groups of diarrheagenic *E. coli*, three other non-*coli Escherichia* species, and various other gram-negative and -positive bacteria found in water. The genotypic assays were performed with hybridization probes generated by PCR amplification of 670- and 623-bp segments of the *gadA*/*B* (GAD) and *uidA* (GUD) genes, respectively. The GAD enzymes catalyze the α-decarboxylation of L-glutamic acid to yield γ-aminobutyric acid and carbon dioxide, which are detected in the phenotypic assay by a pH-sensitive indicator dye. The phenotypic assay for GUD involves the transformation of 4-methylumbelliferyl-β-D-glucuronide to the fluorogenic compound 4-methylumbelliferone. The GAD phenotypic assay detected the majority of the *E. coli* strains tested, whereas a number of these strains, including all representatives of the O157:H7 serotype and several nonpathogenic *E. coli* strains, gave negative results in the GUD assay. Both phenotypic assays detected some but not all strains from each of the four *Shigella* species. A strain of *Citrobacter freundii* was also detected by the GUD assay but not by the GAD assay. All *E. coli* and *Shigella* strains were detected with both the *gadA*/*B* and *uidA* probes. A few *Escherichia fergusonii* strains gave weak hybridization signals in response to both probes at 65°C but not at 68°C. None of the other bacterial species tested were detected by either probe. These results were consistent with previous reports which have indicated that the GAD phenotypic assay detects a wider range of *E. coli* strains than does the GUD assay and is also somewhat more specific for this species. The genotypic assays for the two enzymes were found to be equivalent in both of these respects and superior to both of the phenotypic assays in terms of the range of *E. coli* strains and isolates detected.

United States federal regulations state that public water systems must conduct analyses for fecal coliforms or *Escherichia coli* for any routine drinking water sample that is positive for total coliform bacteria (8). It is recommended in the latest edition of *Standard Methods for the Examination of Water and Wastewater*, 19th ed. (1), that a percentage of presumptive *Escherichia coli*-positive samples be verified. When liquid culture methods such as the multiple tube fermentation method (MTF) are used, good quality assurance requires confirmation of presumptive coliform-positive samples. The recommended method for confirmation of *E. coli* by this procedure is transfer of growth from positive tubes or bottles into EC broth (1) plus 4-methylumbelliferyl-β-D-glucuronide (MUG) and incubation at 44.5°C for 24 h. The transformation of MUG to the fluorogenic compound 4-methylumbelliferone by the enzyme β-glucuronidase (GUD) is considered diagnostic for *E. coli*. When the approved membrane filter method is used, all colonies, up to a total of 10, should be verified. Recommended methods for verification of *E. coli* include the nutrient agar-MUG procedure or other time-consuming classical biochemical tests or commercially available diagnostic kits. This recommendation is based on the finding that 94 to 97% of nonpathogenic *E. coli* strains are reported to contain GUD (12) and that most other bacteria containing GUD do not grow on media selective for coliforms (12, 22).

Recently, considerable attention has been directed toward the use of DNA-based methods for the detection of *E. coli*. Sequences from the *uidA* gene, which codes for GUD, have most frequently been used as probes (2, 5, 10, 23, 32). The *uidA* gene was detected in 97.7% of 435 *E. coli* isolates (21). Approximately one-half of the isolates were from treated waters, and one-half were from raw waters. Another *uidA* probe detected 97% of 116 *E. coli* isolates, 28% of which were found to be MUG negative in a tryptic soy agar medium containing MUG (9). A third study detected 100% of 83 confirmed environmental *E. coli* isolates by a *uidA* probe, of which 90% were GUD positive in tryptic soy agar medium containing MUG (15). Bej et al. suggested that a PCR-based method for *uidA* gene detection appears to identify *E. coli* in a significantly greater number of river water samples than does a MUG-based phenotypic assay (2). These studies indicate that genotypic assays for the *uidA* gene are more sensitive in detecting *E. coli* isolates from water samples than are the corresponding MUG-based phenotypic assays for GUD. They also indicate, however, that a small portion of *E. coli* strains will still be missed by such assays. Other studies have shown that at least a small number of bacteria such as *Shigella* spp., *Escherichia fergusonii*, and *Escherichia vulneris* are detected as false positives in these genotypic assays for *uidA* (6, 16, 26).

Another enzyme, glutamate decarboxylase (GAD), is be-

* Corresponding author. Phone: (513) 569-7332. Fax: (513) 569-7170.

TABLE 1. Phenotypic (enzymatic) and genotypic (hybridization[a]) reactions of bacteria

Culture[b]	No. of strains	No. of strains with positive reaction by:			
		GAD	GUD	*gadB*[c]	*uidA*[d]
Escherichia coli[e]	14	14	14	14	14
Escherichia coli[f]	15	15	15	15	15
Escherichia coli[g]	17	17	0	17	17
Escherichia coli[h]	18	16	0	18	18
Escherichia vulneris	12	0	0	0	0
Escherichia fergusonii[i]	13	0	0	0	0
Escherichia hermannii	12	0	0	0	0
Shigella boydii	15	2	14	15	15
Shigella dysenteriae	10	0	7	10	10
Shigella flexneri	13	9	1	13	13
Shigella sonnei	3	0	3	3	3
Bacteroides fragilis[j]	1	ND[k]	ND	0	0
Clostridium perfringens[j]	1	0	0	0	0
Corynebacterium diphtheriae[l]	1	0	0	0	0
Citrobacter freundii	2	0	1	0	0

[a] Colony and slot blot hybridization results were equivalent.
[b] Except where stated otherwise, all cultures were in-house water isolates. The enterotoxigenic, enteropathogenic, and enterohemorrhagic *E. coli* strains and the *E. vulneris*, *E. fergusonii*, *E. hermanii*, and *Shigella* strains listed above were gifts from the Communicable Disease Center, Atlanta, Ga.
[c] DNA gene probe for the enzyme GAD.
[d] DNA gene probe for the enzyme GUD.
[e] ATCC 11229, ATCC 25922, K-12, and 11 water isolates.
[f] Enterotoxigenic and enteropathogenic.
[g] Enterohemorrhagic.
[h] Eight MUG-negative, GAD-positive cultures were gifts from R. Lum, University of California, Berkeley; eight MUG-negative, GAD-positive cultures were in-house water isolates; and two MUG-negative, GAD-negative isolates were in-house water isolates.
[i] Results from hybridization temperatures at 68°C were negative.
[j] Carol Kreader prepared the DNA from an American Type Culture Collection culture.
[k] ND, not determined.
[l] *C. perfringens* and *C. diphtheriae* were gifts from the bacteriology section of the Ohio State Public Health Laboratory, Columbus.

lieved by some researchers to be more specific to *E. coli* (29, 34). A biochemical assay to detect GAD in gram-negative bacteria was reported in 1974 in which no GAD activity was found in *Salmonella* or *Citrobacter* species (34). Phenotypic assays for GAD have high rates of specificity for *E. coli*, ranging from 97 to 99% (11, 13, 29, 34). Rice et al. (27) have recently published a rapid method for detecting GAD with 95% specificity for *E. coli*. These studies also raise the possibility that a genotypic assay for GAD might further improve upon the specificity of detection of *E. coli* from water samples in comparison with similar assays for GUD. Such an assay should also be expected to show greater quantitative sensitivity than a *uidA* gene probe because it would actually target two highly homologous genes, *gadA* and *gadB*, that appear to always occur in the *E. coli* genome (31).

In the current study, these questions were addressed by the testing of a number of strains of *E. coli*, other *Escherichia* species, and shigellae as well as groups of other common waterborne bacterial species with newly developed probes for both the *gadA*/*B* and *uidA* genes. This is the first report in which a hybridization probe for the *gad* genes has been tested with a variety of bacterial genera.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists some of the cultures used in this study along with the laboratories that supplied and identified them. Other cultures tested are listed below. The positive control for the assays was *E. coli* ATCC 11229. The negative control was *Pseudomonas aeruginosa*. All cultures were stored at −80°C in brain heart infusion (BHI) broth (Life Technologies, Inc., Gaithersburg, Md.) and 9% (vol/vol) dimethyl sulfoxide (Sigma, St. Louis, Mo.). The cultures were grown on BHI agar or in BHI broth at 37°C as needed for DNA extraction for slot blots or colony hybridization.

Standard methods for the presumptive and confirmational identification of *E. coli*. Standard protocols and media for the presumptive detection of *E. coli* included the membrane filter procedure with M-Endo agar, the MTF procedure with lauryl tryptose broth (LTB), and the presence-absence test and broth (1). Confirmation media included EC broth with and without MUG, nutrient agar with MUG (Difco Laboratories, Detroit, Mich.), and the Colilert defined substrate medium (Idexx Corp., Westbrook, Maine) (7, 33). All water isolates isolated in-house were identified by the API 20E System (bioMerieux Vitek, Inc., Hazelwood, Mo.).

GAD and GUD enzyme assays. Cultures were tested for the presence of GAD by the method of Rice et al. (27). Briefly, the substrate L-glutamic acid (Fisher Scientific, Pittsburgh, Pa.) and a pH indicator, bromocresol green (Eastman Kodak Company, Rochester, N.Y.), were added to a pellet of washed cells that had been grown in presence-absence broth or LTB for 22 to 24 h at 35°C. A color change from yellow to blue within 4 h at 35°C was considered a positive response. GUD activity was determined by observation of the cultures under a 366-nm-wavelength fluorescent light after growth on nutrient agar MUG, EC-MUG, or Colilert as previously described (1).

Extraction of genomic DNA. Total genomic DNAs from bacterial cultures were extracted either by a previously reported universal method (14) or with the Insta-Gene DNA purification system (Bio-Rad Laboratories, Hercules, Calif.). DNA concentrations were determined by A_{260} with a Pharmacia Ultro spec II model 4050 spectrophotometer (Pharmacia LKB Biotechnology, Piscataway, N.J.).

Probe construction. Hybridization probes for the *gadA*/*B* and *uidA* genes were generated by PCR amplification of 670- and 623-bp segments of these genes, respectively, from *E. coli* ATCC 11229. The *gadA* and *gadB* genes are 100% homologous in the sequences targeted by the primers and 99.7% homologous over the full length of the amplified regions. Oligonucleotide primers used for these amplifications were identified from previously reported sequences (18, 31), which are listed in GenBank under accession numbers M84024 (*gadA*) and M84025 (*gadB*) and M14641 (*uidA*). Searches for optimal primers were performed with the automated search function of the Oligo 4.1 software program (National Biosciences, Plymouth, Minn.). The forward *gadA*/*B* primer, corresponding to base positions 307 to 324, was 5′-ACCTGCGTTGCGTAAATA-3′. The reverse *gadA*/*B* primer, corresponding to base positions 959 to 976, was 5′-GGGCGGGAGAAGTTGATG-3′. The forward and reverse *uidA* primers were 5′-CCAAAAGCCAGACAGAGT-3′ and 5′-GCACAGCACATCCCCAA AGAG- 3′, corresponding to base positions 1066 to 1083 and 1671 to 1689, respectively. The primers were synthesized on an ABI 381A DNA automated synthesizer (Applied Biosystems, Foster City, Calif.) and purified with oligonucleotide purification cartridges (Applied Biosystems) or by reverse-phase high-performance liquid chromatography with an octylsilyl C_8 column (Aquapore RP-300; Applied Biosystems).

PCR amplifications were performed with a model 480 thermal cycler and GeneAmp PCR reagent kits (Perkin-Elmer, Norwalk, Conn.) according to the manufacturer's directions for the hot start technique with AmpliWax PCR GEM 100 (25). Each 50-μl reaction mixture contained 2.5 mM $MgCl_2$, 200 μM each deoxyribonucleoside triphosphate, 0.5 μM each primer, 0.5 to 1.0 μg of template DNA, and 2.5 U of *Taq* DNA polymerase in 1.25× PCR buffer. Twenty-five step cycles of 1 min each at 94, 58, and 72°C were performed in the thermocycler, followed by a final 7-min extension at 72°C. PCR amplification products were separated by electrophoresis in a 1% agarose gel (Gibco BRL, Gaithersburg, Md.), with Tris-acetate buffer (28) and visualized by staining with ethidium bromide (Fig. 1). Products of the appropriate sizes were identified by comparisons with a 100-bp DNA ladder (Gibco) and recovered from the gel by electrophoresis into NA45-DEAE membranes (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's instructions. After elution from the membranes with a high-salt buffer, a Centricon 100 concentrator (Amicon, Inc., Beverly, Mass.) was used to desalt and concentrate the DNA. One microgram of each DNA fragment was labeled with digoxigenin-11-dUTP by random priming (Genius 1 DNA labeling and detection kit; Boehringer Mannheim, Indianapolis, Ind.) for use as hybridization probes.

Slot blot hybridization and densitometer analysis. Purified 1-, 0.1-, and 0.01-μg DNA samples from each organism were denatured in 4 M NaOH–100 mM EDTA and bound to nylon membranes (Boehringer Mannheim) with a Minifold II apparatus (Schleicher & Schuell) as specified by the manufacturers. Hybridizations were performed overnight at 65 or 68°C with a commercially available hybridization solution (Boehringer Mannheim) according to the manufacturer's instructions (4). The membranes were washed twice for 5 min per wash in 2× sodium citrate buffer (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature and twice for 15 min per wash in 0.5× SSC–0.1% (wt/vol) SDS at 65 or 68°C. Bound probes were detected with the colored substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate according to the instructions of the probe labeling and detection kit manufacturer (4). Each culture

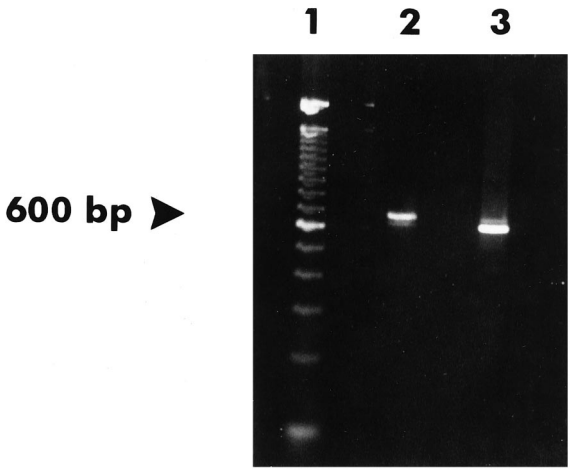

FIG. 1. Gel analysis of *gadA/B* and *uidA* gene fragments used as hybridization probes. Lanes: 1, 100-bp ladder; 2, PCR product from amplification with *gadA/B* gene-specific primers (670 bp); 3, PCR product from *uidA* gene-specific primers (623 bp).

listed in Table 1 and below was tested for hybridization to each probe in at least three independent experiments.

A model 620 video densitometer and the 1-D Analyst II data analysis software (Bio-Rad) were used to quantify the signals obtained in all experiments. Densitometer signals from 1-μg samples of DNA greater than or equal to 0.1 (optical density × bandwidth in nanometers) were considered to be positive responses on the basis of the background signals obtained from a known negative control organism (*Pseudomonas aeruginosa*).

Colony hybridization. Cultures of different bacterial strains grown overnight on BHI agar plates were used to inoculate fresh BHI agar plates in the form of a grid. The inoculated plates were incubated at 37°C for various lengths of time (usually 8 h) until the colonies had grown to approximately 3 to 5 mm in diameter. The colonies were then transferred to nylon filter membranes (Boehringer Mannheim) and lysed, and the DNA was fixed on the membranes as previously described (4). Hybridization and probe detection were performed as described for slot blot hybridizations.

***E. coli* confirmation assays with PCR.** The MTF procedure (1) was performed as a presumptive test for *E. coli* in water samples taken from a local pond. Five aliquots each of 1.0, 0.1, and 0.01 ml of pond water were used to inoculate tubes of LTB, which were then incubated for 24 h at 35°C. One-milliliter aliquots from each tube showing presumptive identification (i.e., growth) were removed, and the DNA was extracted. Extracts containing 1 μg of DNA from each LTB tube were used as templates in PCR amplifications with *gadA/B*- and *uidA*-specific primers. The PCR products were analyzed by gel electrophoresis as described above. GAD and GUD enzyme assays were also performed with aliquots from the LTB cultures described above.

RESULTS AND DISCUSSION

In the current study, the GAD phenotypic assay was found to be positive for a number of *E. coli* strains and isolates that were phenotypically GUD-negative (Table 1). This was true for all 17 of the *E. coli* O157:H7 strains tested as well as 16 wild-type *E. coli* strains from culture collections and water samples. In contrast, only two of the strains examined, both water isolates, were GAD and GUD negative. These results were consistent with data from previous studies that have indicated GAD enzyme activity may be detected in a wider range of different *E. coli* strains and isolates (29, 34).

Until very recently, 100% of enterohemorrhagic *E. coli* strains in the O157:H7 group isolated in the United States have been reported to be GUD negative (9, 17). A study of pathogenic *E. coli* strains indicated that 39% (28 of 71) of enterotoxigenic *E. coli* strains and 25% (2 of 8) of enteropathogenic *E. coli* strains were GUD negative (19). The absence of GUD activity in a number of *E. coli* strains, including those in the O157:H7 group, has been attributed to the production of an enzymatically inactive protein (9). The apparent rarity of GAD-negative *E. coli* isolates may stem from the fact that two independent genes coding for this enzyme, *gadA* and *gadB*, have been found in all strains of this species thus far examined (31). The importance of the GAD phenotype to the metabolism and environmental fitness of *E. coli* remains uncertain. It has been suggested that bacterial decarboxylases of this type may counteract acidic conditions and may serve to control insoluble carbon dioxide (5).

With the exception of one strain of *Citrobacter freundii* which tested positive only for GUD, so-called false positives were observed only within members of the genus *Shigella* (Table 1). Overall, the GAD and GUD phenotypic assays detected 27 and 61% of the 41 *Shigella* strains examined, respectively. Within this group, the two assays gave largely different results depending on the species examined. A majority of *Shigella boydii* (87%) and *Shigella dysenteriae* (70%) strains and all of the *Shigella sonnei* strains tested were detected by the GUD assay, whereas a majority of only the *Shigella flexneri* strains (60%) tested positive by the GAD assay. Only one strain, *S. boydii* C14 2770-51, was detected by both assays, while 17% of the strains tested were not detected by either assay. From a practical standpoint, the detection of shigellae would be an advantage, although in routine water analyses this would be unlikely, since *Shigella* species are either nonlactose fermenters or slow lactose fermenters without the formation of gas. Berger (3), however, reported detection of all four *Shigella* species on the basis of positive GUD reactions from Colilert medium dosed with pure *Shigella* cultures. His recovery rates ranged from 36 to 77%. Further tests would be required to confirm the presence of *Shigella* isolates in Colilert medium inoculated

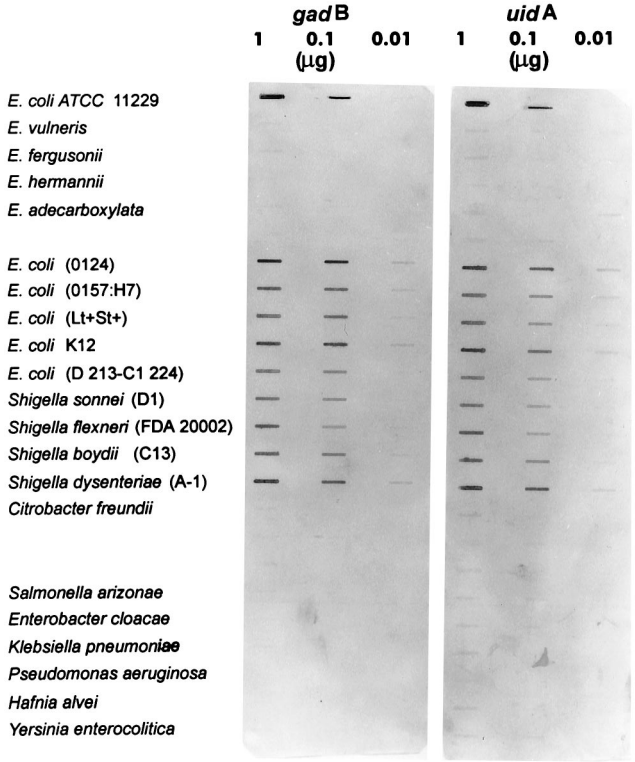

FIG. 2. Slot blot hybridization results for the *gadA/B* and *uidA* probes at concentrations of 1.0, 0.1, and 0.01 μg of DNA isolated from the gram-negative microorganisms listed in Table 1. The positive control was *E. coli* ATCC 11229, and the negative control was *P. aeruginosa*.

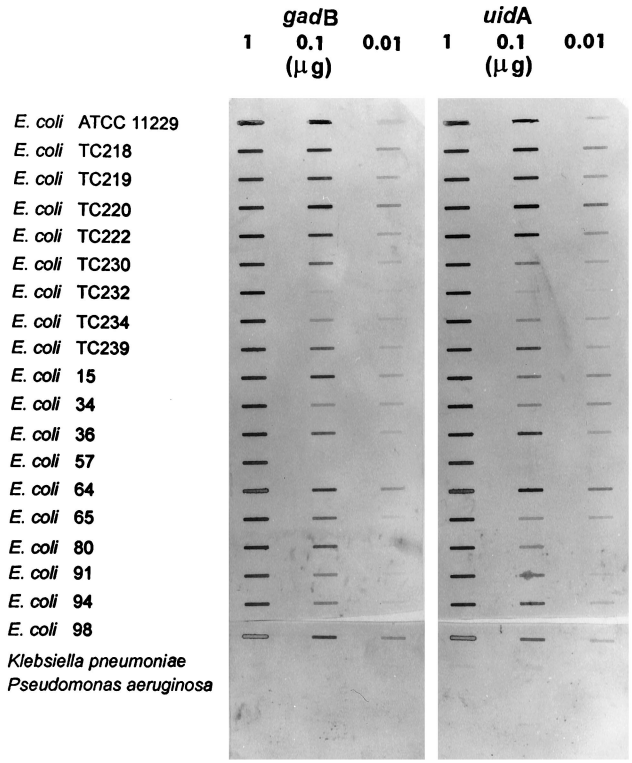

FIG. 3. Slot blot hybridization results for the *gadA/B* and *uidA* probes at concentrations of 1.0, 0.1, and 0.01 μg of DNA isolated from MUG-negative *E. coli*. The positive control was *E. coli* ATCC 11229, and the negative control was *P. aeruginosa.*

with source or distribution system waters. Verification of *Shigella* or enteroinvasive *E. coli* strains from Colilert medium could be accomplished by using a probe specific for these bacteria (30).

All strains of *E. coli* as well as all strains of *Shigella* spp. were detected by both the *gadA/B* and *uidA* gene probes in slot blot hybridization assays (Table 1 and Fig. 2 and 3). These results are consistent with several reports suggesting that gene probes are capable of detecting strains of *E. coli* that may be missed by the corresponding GAD and GUD phenotypic assays (10, 16, 21). Because of this higher level of diagnostic sensitivity, it was not surprising that no differences were observed between the results obtained with the two probes among the limited number of strains examined in the current investigation. Further studies involving the identification and testing of additional GAD- and GUD-negative isolates with both gene probes will be needed to determine the potential advantages of using one or the other or both of these assays for detecting the widest possible range of strains.

As shown in Fig. 2 and 3 and confirmed by densitometry analysis (data not shown), attempts to determine the relative quantitative sensitivities of the two probes in hybridizations with various amounts of target DNAs from the different *E. coli* strains were largely inconclusive. While strong signals were consistently obtained at the 1-μg level of DNA for all *E. coli* strains with both probes, the signal intensities varied significantly, both from strain to strain and from experiment to experiment (data not shown) at lower loadings of target DNAs on the blots. The cause of these variations was not determined.

Strains of *E. fergusonii* were also detected by both probes in hybridizations performed at 65°C, although the signals were consistently weak regardless of the amount of target DNA loaded on the blots. A modest increase in the hybridization temperature to 68°C (Fig. 2 and 3) was found to totally eliminate these signals, with no significant effects on the signals of the other positive strains. Neither the *gadA/B* nor the *uidA* gene probes hybridized to the *C. freundii*, *E. vulneris*, or *H. alvei* strains at either level of stringency or at any level of DNA loading on the blots. Previous studies have indicated that *E. fergusonii*, *E. vulneris*, *Serratia odorifera*, and *H. alvei* may be detected as false positives in gene probe-based tests for *E. coli* (6, 14, 16, 26, 32).

One isolate of each of the following cultures was tested, and all were shown to be both phenotypically and genotypically negative: *Aeromonas hydrophila*, *Enterobacter cloacae*, *Enterobacter intermedius*, *Flavobacterium* spp., *H. alvei*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *P. aeruginosa*, *Salmonella arizonae*, *Salmonella enteritidis*, *Salmonella hirschfeldii*, *Salmonella schottmuelleri*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Yersinia enterocolitica*, and *Vibrio cholerae.*

For the screening of large numbers of isolates, colony hybridizations may be preferable to slot blots. In the current study, no significant differences were observed in results obtained from colony hybridization experiments compared with those obtained by slot blot hybridizations with either probe. An even more rapid and sensitive method of detection would be PCR amplification of *E. coli*-specific DNA sequences. A preliminary examination of this approach was conducted with the *gadA/B*- and *uidA*-specific primers developed in this study to provide confirmational detection of *E. coli* in pond water samples after a presumptive assay by the MTF procedure. Both PCR assays confirmed *E. coli* in tubes inoculated with 0.1 ml of pond water on the basis of production of the appropriate DNA bands in electrophoresis gels, whereas the GAD and GUD phenotypic assays confirmed the presence of *E. coli* only in tubes incubated with 1 ml of pond water. This indicated that the sensitivity of the PCR assay was at least 10-fold greater in this format. Similar assays of DNA extracts taken directly from water samples have been reported (24). While such assays have the potential to provide optimal sensitivity of *E. coli* detection, appropriate controls must be included to assess the possible effects of inhibitory compounds on the results of the amplifications (16, 20), and they do not necessarily address the question of viable versus nonviable organisms in the samples.

The results of the current investigation indicate that genotypic assays for either GAD or GUD should provide superior confirmational detection of *E. coli* in drinking water samples compared with the corresponding phenotypic assays for these samples. In contrast to the phenotypic assays, the choice of the *gadA/B* or *uidA* genes as a target for such genotypic assays may be relatively unimportant, but the use of both targets (for example, in a multiplex PCR) could further increase the specificity and detect the widest range of *E. coli* strains possible. At present, the major impediments to incorporating such assays into routine water analyses appear to be their cost and their requirements for technical expertise. With the growing number of laboratories and personnel gaining expertise in the field of molecular biology and the development of relatively inexpensive kits by various companies for performing such assays, these impediments may be eliminated in the near future.

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